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The molecular biology of circadian clocks

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Being in time: the importance of post-translational processes in circadian clocks

by Dr Jan C. Schöning and Dr Dorothee Staiger

The well-known daily variations in behaviour, physiology and metabolism are generated by endogenous timekeepers known as circadian clocks. The core clockwork is an oscillator that keeps an approximate 24-hour rhythm and is synchronised to the outside day mainly by environmental light. This central oscillator operates at the level of a single cell, generating circadian rhythms of clock gene expression through negative transcriptional feedback. Accumulating evidence points to the importance of post-translational processes for the exact timing of clock protein oscillations. Output pathways then translate the protein cycles into physiological and molecular rhythms, directing these processes to an appropriate time of the day.

Circadian rhythms (from the Latin "*circa diem*", about one day) have a profound impact on organisms by providing a temporal structure for biological processes. These range from physiology and behaviour, e.g. the sleeping-waking cycle, down to biochemical processes in organs and daily cycles of gene expression patterns in individual cells. The underlying biological timekeeper is made up of three components. A core clockwork that is a self-sustained oscillator produces a rhythm of about 24 hours [1]. Input pathways synchronise the oscillator to the day outside via perception and integration of environmental cues, predominantly light and temperature. The third component is output pathways that impart the 24-h rhythms onto physiology and behaviour [Figure 1]. In this review we discuss basic principles of circadian clocks as well as new insights into the complex interactions of clock proteins that contribute to the maintenance of the 24-h period.

It's all in the genes

In the early days of chronobiology, the daily phases of locomotor activity and rest served as circadian readout, just as the hands of a clock could provide a way of monitoring the invisible, unknown underlying clockwork. Pioneering advanced genetic approaches have been instrumental in proving unequivocally that circadian clocks have a genetic basis (rather than relying on a magic cosmic factor X).

To identify gene products involved in timekeeping, in the early 1970s Konopka and Benzer carried out chemical mutagenesis of the fruitfly *Drosophila melanogaster* and screened the offspring for alterations in circadian period, indicating abnormal biological timing [2]. Flies were isolated with either a long period of ~28h (per^{L}), a short period of ~19h (per^{S}) or no rhythmicity at all. Interestingly all mutants mapped to a single locus, known as *Period*.

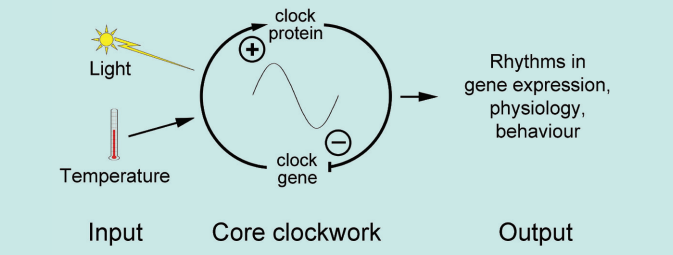

Figure 1. Conceptual model of the circadian system (see text for details). Additional interactions such as feedback of clock-regulated components on the input, are omitted for simplicity.

About 20 years later, the *Period (Per)* gene was the first clock gene to be cloned. Its temporal expression pattern already suggested a simple model for the generation of circadian rhythms: the transcript of *Period* accumulates over the day with a peak level early at night, while the protein reaches its highest level 4h later. The protein in turn inhibits transcription of its own gene, and turnover of the repressing protein allows the cycle to be restarted [3,4]. The challenge of the subsequent years was to unravel how this simple negative feedback loop takes about 24 hours to close.

It soon became obvious that PER does not do the job alone. A mutation at the *Timeless (Tim)* locus eliminates circadian rhythms of behaviour as well as molecular rhythms of the *Per* transcript [5]. The molecular characterisation of *Tim* provided evidence that TIM may be a partner of PER in a two-component circadian clock: *Tim* RNA cycles in phase with *Per*, and *Per* and *Tim* oscillations both depend on intact PER and TIM. Furthermore, the tim^{01} mutation blocks nuclear uptake of PER protein. In addition, PER and TIM have been shown to physically interact, leading to the idea that PER and TIM are taken up into the nucleus as a heterodimeric complex [5].

In 1998, yet another type of clock protein was identified that contained basic helix-loop-helix DNA binding motifs and PAS protein interaction domains. This group of proteins includes dCLK that is a paralogue of mammalian circadian locomoter cycle kaput, CLOCK, (i.e. dCLK has a gene sequence that has a common ancestor with CLOCK) and CYCLE (CYC). These proteins are important additional factors in the maintenance of circadian rhythmicity in *Drosophila melanogaster* [6,7]. Together, these four proteins make up a core circadian oscillator in which dCLK and CYC act as positive factors to activate the transcription of the negative factors PER and TIM which subsequently feed back to inhibit the positive factors.

Over the years, it has become clear that this seemingly simple scenario involves an intricate network of protein-protein interactions and post-translational events [8]. Starting in the middle of the day, dCLK and CYC bind as heterodimer to the *per* and *tim* promoters to switch

on transcription. PER and TIM proteins in turn shut off transcription by inhibiting dCLK-CYC. This inhibition is accomplished by direct contact between PER-TIM and dCLK-CYC, interactions of which interfere with dCLK-CYC binding to the *Per* and *Tim* promoter regulatory elements [9].

Furthermore, phosphorylation of the clock proteins serves both to fine-tune their activity and regulate their half-life. PER is the target of the kinase DOUBLETIME, and phosphorylation of PER in the nucleus increases PER´s ability to act as transcriptional repressor [10]. Similarly, dCLK oscillates between a hypophosphorylated and a hyperphosphorylated form [9]. At the same time as the formation of a complex between PER and dCLK, which inhibits the transcriptional activator function of dCLK-CYC, the phosphorylation of dCLK increases. Continued phosphorylation of both dCLK and PER by DBT Kinase also promotes their degradation. Once PER and dCLK are depleted, hypo-phosphorylated dCLK accumulates in parallel with *Per* and *Tim* mRNA, suggesting that the hypophosphorylated form of dCLK is the transcriptionally active one [9].

How is the feedback loop stretched to 24h?

The alternating phases of cytoplasmic PER accumulation and the nuclear repression of dCLK-CYC activity that leads to a decline in PER and TIM, have long been thought to be separated by the time it takes for PER and TIM to associate in heterodimers that are then taken up into the nucleus. More specifically, the lag phase of PER protein accumulation relative to its mRNA is thought to result from the phosphorylation of PER by DBT, promoting PER turnover. Binding of TIM would then contribute to the stabilisation of PER, thus allowing it to build up to a significant level and ultimately move into the nucleus [8].

Recent findings from the Young laboratory indicate that the 24-h period of the oscillator may in fact rely on different means of temporal PER and TIM regulation [11]. To follow the fate of PER and TIM in single cells, the fluorescence resonance energy transfer (FRET) technique was used .

FRET has emerged as a new and powerful technique for monitoring protein-protein interaction *in vivo*. It uses energy transfer from the fluorescent reporter protein CFP to YFP which only occurs if the two reporter proteins come into close contact by virtue of interaction of their fusion partners [Figure 2].

Upon transient expression in *Drosophila* S2 cells, a PER-CFP fusion protein is completely located in the cytoplasm while a TIM-YFP fusion protein is mostly located in the cytoplasm. However, this changes upon co-transfection of PER and TIM. As expected according to currently accepted understanding of the system, heterodimerisation of PER and TIM in the cytoplasm can be shown by the occurrence of FRET. Surprisingly, however, this physical association was detected as early as half an hour after onset of PER and TIM synthesis. Moreover, the heterodimer accumulated in discrete cytoplasmic regions. This contradicts the former assumption that a lag in PER-TIM heterodimerisation is responsible for the time delay between PER/TIM expression and nuclear uptake and thus dCLK/CYC repression.

After several hours PER and TIM can be detected in the nuclei but FRET decreased upon translocation. In most of the transfected cells PER accumulated in the nucleus earlier or at higher rates. These findings imply that PER and TIM are transported to the nucleus independently. This result contradicts completely the former assumption that PER's nuclear uptake and activity requires the heterodimeric complex with TIM. Nevertheless it is still assumed that TIM promotes the nuclear uptake of PER [Figure 3].

The modified view of the *Drosophila* clock is supported by investigations with the per^{L} mutant. Its period-lengthening effect has been attributed to delayed nuclear uptake of the PER-TIM heterodimer due to a poorer association between PER^{L} and TIM, as observed in yeast two hybrid assays [5]. However, Meyer *et al* did not detect alterations in PER-TIM heterodimerisation kinetics in PER^{L}-expressing S2 cells, but rather an extremely long lag of about 9 hours for the nuclear translocation of both proteins [11]. Thus, delayed association between PER and TIM before translocation into the nucleus cannot be responsible for the 24-h period of the negative feedback loop. These findings are in accordance with the suggestion of Nawathean and Rosbash that rhythmic PER phosphorylation does not serve as a control element for the nuclear uptake but rather for its activation [10]. Phosphorylated PER that is active as a repressor of dCLK-CYC is retained in the nucleus, whereas non-phosphorylated, inactive PER undergoes nuclear import and export.

Taken together, PER stability in the cytoplasm and its nuclear uptake would neither depend on its phosphorylation state nor on heterodimerisation with TIM. Rather, nuclear uptake of PER and TIM is delayed by an as yet undescribed timer. This timer is itself influenced by PER, suggesting an elusive function of PER in the cytoplasm. As both studies have been performed in the *Drosophila* Schneider 2 cell line, which is derived from late stage embryos but is not rhythmic itself, it will be important to repeat the above approaches in whole flies. Apart from autoregulating their rhythmic expression within the circadian oscillator, clock proteins also convey rhythmicity upon behaviour,

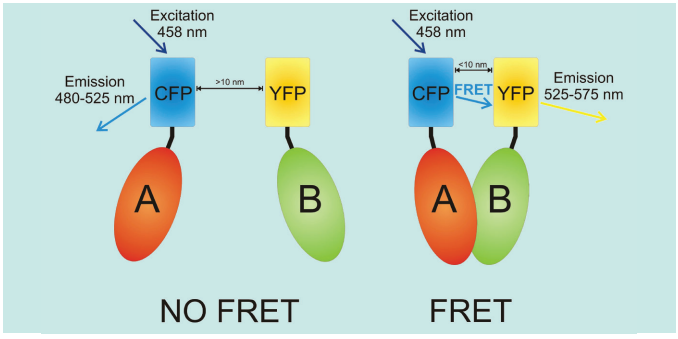

Figure 2. Principle of FRET (fluorescence resonance energy transfer). Two proteins of interest (A and B) are expressed as a fusion with CFP and YFP, respectively (left). Interaction of the fusion proteins can be examined after excitation with light of 458nm wavelength. If the distance between the two interaction partners is less than 10 nm FRET occurs (right) and light of 525 to 575 nm is emitted instead of the normal CFP emission (480-525 nm).

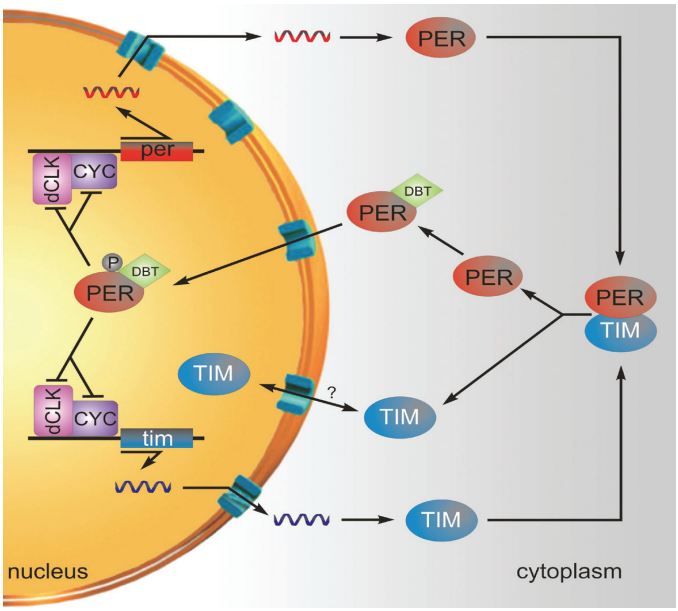

Figure 3. Model of the core clockwork in Drosophila: The transcription factors dCLK and CYC activate transcription of PER and TIM which shortly after translation form a heterodimer. About 6 h later PER and TIM dissociate and enter the nucleus. The phosphorylated and thus activated form of PER remains in the nucleus and represses the function of dCLK and CYC. TIM is thought to shuttle between cytoplasm and nucleus promoting the uptake of PER [16, with modifications].

involved in basic cellular functions in *Drosophila* that are under control by dCLK suggests that clock proteins transduce timing signals mostly by transcriptional regulation of target genes [12].

One protein - two functions

Intriguingly, post-translational modification by phosphorylation has been shown lately to completely change the function of a clock protein from transcriptional inhibition within the nucleus to the promotion of protein accumulation in the cytoplasm. In the bread mold *Neurospora crassa*, the core clockwork comprises the clock protein Frequency (FRQ) as well as the PAS domain white collar 1 and 2 proteins. The WC proteins, as a white collar complex (WCC), activate *frq* transcription. FRQ protein then feeds back to inhibit wc-1 transcription. Later during the circadian cycle FRQ promotes accumulation of WCC at the post-translational level in the cytoplasm. This conversion from a nuclear repressor into a cytoplasmic activator is caused by phosphorylation of FRQ at a PEST (proline, glutamate, serine, and threonine rich) site, showing that post-translational modification by phosphorylation can completely change the function of a clock protein [13].

No transcriptional feedback in cyanobacteria?

Notably, the cyanobacterium *Synechococcus elongatus* provides the first example of a post-translational oscillator. Although the cyanobacterial KaiA, KaiB and KaiC clock proteins make up a molecular feedback loop similar to the one in flies, the oscillator function relies on protein interaction among the clock proteins rather than on transcriptional regulation [14,15]. In cultures grown in constant darkness transcripts of the three Kai clock genes do not accumulate to detectable levels and the Kai clock protein abundance is constant. Nevertheless, a continuous oscillation of the KaiC phosphorylation state is observed [14]. Taking this a step further, Kondo and co-workers have been able to reconstitute the self-sustained oscillation of KaiC phosphorylation *in vitro* by solely combining the KaiA, KaiB and KaiC proteins in the presence of ATP [15].

This opens up one way to investigate molecular details of the time-keeping mechanism by studying protein-protein interactions of Kai protein variants.

Outlook

After a long period of focus on mechanistic details of the transcriptional feedback loop, the spotlight is now being turned on biochemical aspects of clock protein function such as pools of active and inactive proteins and cytoplasmic and nuclear functions rather than overall protein levels.

Taken together these new observations, summarised in part above, highlight how post-translational events influence the level, subcellular distribution and activity of clock proteins. In the end, only the complex cooperation of transcriptional and different forms of post-transcriptional and post-translational control leads to a closed loop of gene expression which feeds back in 24-h intervals.

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The authors

Jan C. Schöning, Ph.D.
Dorothee Staiger, Ph.D.
Address for correspondence:
Jan C. Schöning,
Molecular Cell Physiology, University of Bielefeld, D-33615 Bielefeld, Germany.
Telephone: +49 0521 106 5600
Fax: +49 0521 106 6410
e-mail: j.schoening@uni-bielefeld.de